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TITLE OF THE INVENTION (500 characters max)

Xylogucan Conjugates Useful for Modifying Cellulosic Textiles

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Respectfully submitted,

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PROVISIONAL APPLICATION FOR LETTERS PATENT

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XYLOGUCAN CONJUGATES USEFUL FOR MODIFYING CELLULOSIC TEXTILES

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XYLOGLUCAN CONJUGATES USEFUL FOR MODIFYING CELLULOSIC TEXTILES

FIELD OF THE INVENTION

The textile industry is the primary beneficiary of the technological invention described in this patent application. The invention relates to the use of xyloglucan conjugates as molecular anchors for attaching functional chemical groups to cellulose, in particular, the cellulose fibers contained in textiles.

BACKGROUND OF THE INVENTION

Dyes used in the textile industry are classified according to the way they are applied to the fiber. The Color Index (C.I.) lists 19 different dye classes known as "application ranges." Of the 19, only 5 are of significance for the dyeing of cellulosic fibers [Waring, D.R. (1990) "Dyes for cellulosic fibers" In: The Chemistry and Application of Dyes (D.R. Waring and G. Hallas, eds.) pp. 49-106, Plenum Press, New York]. These are vat, sulfur, direct, reactive, and azoic dyes.

Vat and sulfur dyes are water-insoluble colorants that are converted into an alkali-soluble (leuco) form by a reduction process. After the leuco form is absorbed to cellulose, it is reoxidized and trapped in the fiber. Vat dyes suffer from a high cost of production and application, and sulfur dyes are limited to dull hues. These dyes are therefore steadily losing commercial value.

Direct dyes are water-soluble colored compounds that are applied to the substrate fiber directly, that is, without chemical manipulation. Direct dyes rely on their affinity for cellulose

("substantivity") through non-covalent binding. Salt (1-5 g/l NaCl) is usually added to the dye solution to improve application efficiency. Direct dyes must be sufficiently soluble in water to enable enough dye to bind to the fiber to provide the desired color intensity. Thus direct dyes usually are characterized by poor wash fastness.

Reactive dyes are colorants that contain a reactive group capable of forming a covalent bond with the hydroxyl groups of cellulose. Accordingly, these dyes exhibit excellent wash fastness. However, the dyeing process is carried out in water, which competes with cellulose in the reaction with the dye, often leading to poor fixation efficiencies. The fixation is improved by employing very high salt concentrations (50-100 g/l NaCl), but even then the loss of the unfixed dye due to hydrolysis ranges from 20 to 50 %. The large amount of unfixed dye makes extensive washing of the dyed fabric necessary, leading to a large volume of wastewater.

A majority of direct and reactive dyes belong to the class of azo dyes, *i.e.*, they contain the -N=N- linkage [Stead, C.V. (1990) "Chemistry of azo colorants" in Colorants and Auxiliaries 1:146-195]. Azo dyes are synthesized by reacting an aromatic amine with nitrous acid to form a diazonium salt ("diazotization") (see Fig. 1). The azo linkage is generated from the diazonium salt by coupling it with an electron-rich aromatic compound ("coupling component"), most commonly an aminonaphthol or an aminonaphthalenesulfonic acid.

The azoic dyeing process makes use of coupling components that have substantivity for cellulose. The fabric is impregnated with the coupling component and then treated with a diazo component. The resulting azo dye is highly insoluble and binds non-covalently to cellulose. The diazo components that are normally formed in a diazotization reaction are unstable compounds that have to be prepared immediately before the coupling step. This presents the obvious disadvantage that diazotization must be carried out in the dye house. This has been alleviated to some degree by producing stable derivatives ("fast salts") that liberate the reactive diazonium salt upon dissolution in water or chemical activation [Stead, C.V. (1990) *supra*]. The use of azoic dyes has declined dramatically in recent years.

The reactive dyes commonly used today comprise the class with the best fastness properties. However, these dyes generally are expensive, having the poorest application efficiency of any class of dyes. Typical efficiency of fixation of reactive dyes on cotton is only 50-80%; thus, depending on the particular dye, 20-50% is wasted. In summary, the dyeing of cellulose fabrics is plagued by intrinsic problems that cannot be solved completely within the framework of conventional methods. Dyes that bind non-covalently to cellulose have to strike a balance between the opposing characteristics of solubility and substantivity, and those that bind covalently, i.e. reactive dyes, suffer from poor application yields and the need to cope with excessive amounts of waste dye, salt, and water.

Xyloglucan is a hemicellulosic polysaccharide (Fig. 2) that is a major component (20-40%) of the primary cell walls of a wide range of plants [Hayashi, T. (1989) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 40:139-168]. Primary cell walls encase growing cells and the cells of the succulent tissues of plants. Primary cell walls are not lignified; lignification is a characteristic of secondary cell walls, which are the characteristic cell walls of woody tissues. Most of the xyloglucan in primary cell walls is bound tightly to the surface of cellulose microfibrils *via* multiple hydrophobic interactions and hydrogen bonds [Valent and Albersheim (1974) *Plant Physiol.* 54:105-108; Whitney *et al.* (1995) *Plant J.* 8:491-504]. Strong alkali (~4 N KOH) is required to solubilize a majority of cellulose surface-bound xyloglucan. Although xyloglucan binds to cellulose almost instantaneously *in vitro*, xyloglucan is highly water-soluble when it is not bound to cellulose.

Xyloglucan functions in primary cell walls as a flexible cross-link between rigid cellulose microfibrils to form a strong, dynamic network that controls cell growth and thereby is believed to control the shapes and sizes of encased cells [Hayashi, T. (1989) *supra*; Carpita and Gibeaut (1993) *Plant J.* 3:1-30; Pauly *et al.* (1999) *Plant J.* 20:629-639]. The cellulose/xyloglucan network spontaneously assembles when newly synthesized cellulose and xyloglucan come together at the outer surface of the cell membrane. This process occurs because xyloglucan is highly water-soluble yet bind tightly to the cellulose surface immediately upon contact. The interaction of xyloglucan with cellulose plays a key role in controlling the growth of plant cells

because it has the requisite physical properties of high solubility in water and avid binding to cellulose.

The valuable structural properties of xyloglucan, as with any polymer, arise as a consequence of its chemical structure [Vincken *et al.* (1997) *Plant Physiol.* 114:9-12]. Xyloglucan is structurally related to cellulose in that xyloglucan has a "cellulosic" backbone, that is, the backbone is composed of 1,4-linked ß-D-glucopyranosyl (Glc*p*) residues. Xyloglucan is highly branched, with three out of four of the Glc*p* residues of most xyloglucans bearing side chains attached to O-6. Each of the side chains is composed of from 1 to 3 glycosyl residues. The side chain glycosyl residue attached directly to the backbone is almost always ß-D-xylopyranosyl (Xyl*p*). In seed xyloglucans [York *et al.* (1993) *Carbohydr. Res.* 248:285-301], a terminal ß-D-galactopyranosyl (Gal*p*) residue is attached to O-2 of many of the ß-D-Xyl*p* residues. Seed xyloglucans are the focus of this invention disclosure due to their ease of extraction, chemical and physical properties, availability in large quantities, and low cost.

The side chains of xyloglucans have profound effects on their physical properties. For example, complete removal of the side chains would produce cellulose, which is completely insoluble. Removal of some of the galactosyl residues (while leaving the underlying xylosyl residues in place) increases the viscosity of the polymer, eventually leading to gel formation [Shirakawa *et al.*, (1998) *Food Hydro colloids* 12:25-28]. The rheological properties of the polymer are also affected by its molecular weight. The viscosity increases and the solubility decreases as the molecular weight of the xyloglucan increases.

The galactosyl content and molecular weight of xyloglucan can be manipulated using readily available enzymes. Galactosyl residues can be removed by fungal ß-D-galactosidases [Reid *et al.* (1988) Enzymatic modification of natural seed gums in Gums and Stabilizers for the Food Industry 4, G.O. Phillips, D.J. Wedlock and P.A. Williams, eds. p. 391, IRL Press, Oxford, England; York *et al.* (1993) *supra*]. The molecular weight can be decreased by treatment with any of several fungal ß-D-*endo*-1,4-glucanases, which cleave the glycosidic linkages of the regularly-spaced, unbranched ß-D-Glc*p* residues in the xyloglucan backbone (see Fig. 2) [York *et al.* (1993) *supra*; Pauly *et al.* (1999) *Glycobiology* 9:93-100]. The unbranched, 4-linked,

ß-D-Glc*p* residues are located every fourth residue of the ß-D-glucan. If the *endo*glucanase digestion of xyloglucan is carried out to completion, oligosaccharide subunits consisting of 7 to 9 glycosyl residues are generated (the number of residues per subunit depends on the length of the of the side chains) [York *et al.* (1990) *Carbohydr. Res.* 200:9-31]. This collection of oligosaccharides is called S_1, i.e., each S_1 oligosaccharide is a *single subunit* with four glucosyl residues in its backbone. Larger oligosaccharides are produced when the digestion is incomplete. For example, a collection of *endo*glucanase-generated xyloglucan oligosaccharides with from 14 to 18 residues is called S_2. Each S_2 oligosaccharide consists of two S_1 subunits linked together by a ß-1,4-D-glucopyranoside linkage.

The seeds of a number of different legumes have been shown to contain large amounts of water-soluble xyloglucan [Kooiman, P. (1961) *Res. Trav. Chim.* 80:849-865], which provides a huge natural resource for the preparation of the xyloglucan conjugates disclosed herein. Most of the xyloglucan used in commercial processes comes in the form of tamarind kernel powder (TKP) prepared from the dried seeds of *Tamarindus indica*, a tropical legume. TKP, which is widely used in the textile industry, especially in Asia, typically is composed of approximately 60% xyloglucan, [Shankaracharya, N.B. (1998) *J. Food Sci. Technol.* 35:193-208]. For example, TKP is commonly used as a sizing agent during textile manufacturing. Sizing agents are applied as an aqueous solution to warp yarns in order to strengthen and lubricate them, thereby increasing the efficiency of the weaving process and improving the quality of the resulting fabric.

TKP has two major advantages over starch as a sizing agent: it is cheaper and it can be applied in smaller amounts to obtain similar results [Shankaracharya, N.B. (1998) *supra*]. TKP is also used as a thickener to prevent the spreading of dye during fabric printing. A patent (Racciato, 1982, U.S. Patent No. 4,324,554) has been granted for the use of TKP as a dye antimigrant. Antimigrants are water-soluble polymers that inhibit the movement of dye particles through the capillary structure of textile fabrics during the drying process, leading to uneven deposition of dye on the fabric. Antimigrants are one of the components of virtually every formulation used for dyeing cotton as well as in continuous application processes used in the manufacture of fabrics composed of polyester/cotton blends.

GB948678 discloses a process for dyeing or printing of textiles using polysaccharides to which dye molecules are covalently linked. However, the dyeing is effected by addition of non-carbohydrate resin precondensates that are polymerized by high temperature curing. This is necessary because the polysaccharides included in this disclosure do not have strong affinity for cellulose.

U. S. Pat. No. 6,225,462 discloses a composition comprising a polysaccharide conjugate wherein a protein is covalently attached to xyloglucan to anchor it to the cellulosic fabric. The described use of the composition is an additive in laundering, and is not intended as permanent modification. The attached protein is specified to have a molecular weight of at least 5000.

Both U. S. Pat. No. 6,225,462 and EP0930334 disclose a polysaccharide conjugate as carrier for small molecules, such as fragrances or dyes, but these are only physically adsorbed and not covalently attached to the polysaccharide and thus would not be "permanently" linked to the fabric.

Due to the limitations of the conventional dyeing methods mentioned above, there is a need in the field for a new method of dyeing that is simple, more efficient, economical and environmentally safe. Towards this end, the present application discloses new methods of dyeing cellulosic material by employing xyloglucan conjugates.

SUMMARY OF THE INVENTION

The present invention provides xyloglucan conjugates that are useful for attaching a variety of functional chemical groups to cellulosic material. The term, "cellulosic material" as used in the present invention means any material, which is wholly or partly, made of cellulose. Examples of such material include but are not limited to paper, pulp products, and cellulosic fabrics. In the context of the present invention a cellulosic fabric is any cellulose-containing fabric known in the art, such as cotton, viscose, rayon, ramie, linen, Tencel®, or mixture thereof,

or mixtures of any of these fibers, or mixtures of any of these fibers together with synthetic fibers or wool such as mixtures of cotton and spandex (stretch denim), Tencel® and wool, viscose and polyester, and cotton and wool. Paper or pulp products include lignin-containing materials such as particleboards, fiberboard, and paper.

The xyloglucan conjugates of the invention are composed of oligosaccharides ranging in size from two to approximately one hundred glycosyl residues that have a functional group covalently attached to their reducing end. The functional groups that can be attached to the xyloglucan conjugates include, but are not limited to, dyes, fabric softeners, water and oil repellants, antimicrobial agents, antisoiling agents, soil release agents, stain release agents, firming agents, or lubricants. The xyloglucan conjugates of the invention bind spontaneously, specifically, and so avidly to cellulose that the xyloglucan serves as a molecular anchor for the chemical covalently attached to the reducing end of each xyloglucan oligosaccharide. Specifically exemplified herein is a xyloglucan conjugate having a dye molecule (e.g., azo dye) that can be used to dye cellulosic material (e.g., cotton). This method of dyeing is economical, environmentally safe, and offers a large variety of colors that are durable and color fast.

The invention also provides methods of preparing a variety of xyloglucan conjugates. Typically, the glycosidic bonds of xyloglucan polymers are partially hydrolyzed (cleaved) with enzymes to generate xyloglucan oligosaccharide (XGO) fragments ranging in size from two to approximately one hundred glycosyl residues. The enzymes useful for catalyzing such hydrolysis reactions are *endo*glucanases, which can be readily isolated from plants or prepared by employing recombinant technology available in the art. A functional group is then covalently attached directly to the reducing end of the oligosaccharide fragments to yield the xyloglucan conjugates. Alternatively, xyloglucan polymers are exhaustively digested with enzymes to generate oligosaccharide fragments ranging in size from two to twenty glycosyl residues. A functional group (e.g., a dye molecule) is then attached covalently to the oligosaccharides using one or more of various chemical reactions disclosed herein to generate a desired xyloglucan conjugate. Finally, the resulting xyloglucan conjugates are linked to larger xyloglucan fragments using the enzyme xyloglucan endotransglycosylase.

The xyloglucan conjugates of the invention are useful in a variety of applications depending upon the particular functional group attached thereto. We have used as our primary example in this application the ability of xyloglucan conjugates, each containing a dye molecule to dye used for dyeing fabrics. Examples of the utilities of other functional groups include fabric softeners, water and oil repellants, antimicrobial agents, antisoiling agents, soil release agents, stain release agents, firming agents, and lubricants.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a scheme showing the synthesis of azo dyes.

Fig. 2 shows the structure of Tamarind Seed Xyloglucan. Arrows indicate glycosidic bonds that are susceptible to attack by *endo*glucanase and xyloglucan endotransglycosylase.

Fig. 3 shows the action of xyloglucan endotransglycosylase (XET). Two different xyloglucan substrates are distinguished by their shading. Each oligosaccharide subunit is indicated by a rectangle.

Fig. 4 is a scheme illustrating how to prepare and use xyloglucan conjugates of the invention. Xyloglucan subunit oligosaccharides are indicated by rectangles. Dye or other functional groups are indicated by asterisks.

Fig. 5 is a scheme showing the synthesis of XGO-dye conjugates.

Fig. 6 shows examples of electrolytic oxidation of XGO and amide bond formation.

Fig. 7 shows condensation of XGO with pyrazolinones.

DETAILED DESCRIPTION OF THE INVENTION

The inventors took advantage of the property of xyloglucan to bind spontaneously and avidly to the surface of cellulose to develop a new method of dyeing that alleviates problems associated with the methods that are currently used. In this application, xyloglucan serves as a molecular anchor for binding, to a cellulose-containing material, a chemical with a desired function (e.g. dye, see Fig. 1). For example, functional groups that are covalently attached to the reducing end of xyloglucan fragments rapidly and strongly adhere to the surface of cellulose-containing textiles (cotton, rayon, flax). For example, dyes that are covalently attached to xyloglucan or xyloglucan fragments are rendered highly soluble in aqueous solution, but the xyloglucan-dye conjugate binds strongly upon contact to the surface of cellulose fibers. This minimizes the loss of dye due to incomplete binding or to competing processes, such as unwanted chemical reactions, precipitation, diffusion, or binding to other surfaces. In addition to improving the efficiency of the dyeing process, this approach will reduce contamination of the environment by functional molecules (e.g. dyes) that do not bind to the fabric. Furthermore, any functional molecule, such as a dye, that is covalently attached to a xyloglucan fragment that fails to bind to the fabric can be removed from the waste stream simply by bringing it into contact with cellulose, which is an inexpensive and extremely abundant material.

The use of the xyloglucan-conjugates of the invention is not limited to the dyeing process. Covalent modifications of the reducing end of xyloglucan fragments allow a variety of functional groups to be anchored to the surface of cellulose-containing materials. The functional groups that can be attached include molecules that soften or firm up the fabric, lubricate the fabric, make the fabric susceptible to staining, endow the fabric with antimicrobial properties, or render the fabric with resistance to water or oil.

We prepare xyloglucan conjugates, in this invention, by a combination of chemical and enzymatic methods. In one embodiment of the invention, xyloglucan polymer is digested partially by an *endo*glucanase to produce a mixture of xyloglucan oligosaccharides varying in size from two to one hundred glycosyl residues (Fig. 4). The functional chemical entity (e.g.

dye, see Fig. 3) is then attached chemically to the reducing ends of the collection of xyloglucan oligosaccharides (Fig. 4).

Alternatively, xyloglucan conjugates are prepared by employing the following sequence: first, the xyloglucan polymer is digested completely into S_1 fragments by treatment with *endo*glucanase (Fig. 4). The S_1 oligosaccharides are then chemically functionalized by reaction with the appropriate chemical to give an S_1-conjugate. An enzyme called xyloglucan endotransglycosylase (XET) [Cosgrove, D.J. (1999) *Ann. Rev. Plant Physiol. Mol Biol.* 50:391-417] is then used to link the S_1 conjugates to xyloglucan fragments of intermediate size (two to one hundred glycosyl (sugar) residues).

XET is similar to *endo*glucanase in that it cleaves polymeric xyloglucan by attacking the unbranched glucosyl residues in the backbone (see Fig. 3). However, XET does not catalyze hydrolysis of the polymer. Rather, it catalyzes the formation of a new glycosidic linkage, attaching one of the fragments to the non-reducing end of another xyloglucan molecule. Therefore, XET can be used to simultaneously reduce the molecular weight of the polysaccharide and attach chemically modified xyloglucan oligosaccharides to the ends of the resulting xyloglucan fragments. An example is provided below that illustrates how XET can be used to generate xyloglucan fragments that have a dye or other surface-modifying agent attached to the reducing end.

Due to the fact that XET transfers another carbohydrate molecule instead of water, the total number of carbohydrate molecules remains constant throughout the reaction. Initially, the reaction mixture contains only very large xyloglucan ($>10^5$ Da) and relatively small S_1-dye ($\sim 10^3$ Da) molecules. As the reaction proceeds, the large molecules are cut and capped off with S1-dye molecules. This process continues until equilibrium is reached where the size distribution ceases to change. The size distribution will be centered around a molecular weight that is determined by the initial mass ratio of xyloglucan to S_1-dye. For example, if that ratio is 4:1, the average molecular weight of the product will be equal to that of S_5. The polydispersity of the products will thus depend on the reaction conditions, but will generally decrease as the reaction progresses. The final size dispersion will be governed by the maximum entropy of the system.

In order to produce various chemically modified xyloglucan fragments that are small enough to have low viscosity, high solubility, and a high content of the chemical adduct, yet maintain their ability to bind to cellulose, the inventors took advantage of the recombinantly expressed enzymes such as galactosidase and *endo*glucanase. By judicious use of enzymes that have been cloned, over-expressed and purified, the physical properties of the xyloglucan fragments can be tailored as desired. These two enzymes have roughly opposite effects on the rheology of xyloglucan so its physical properties can be adjusted by using both enzymes in the appropriate proportion.

As described above, xyloglucan binds spontaneously and avidly to the surface of cellulose microfibrils. Xyloglucan's strong affinity for cellulose can be utilized in order to impart a broad range of desirable properties to cotton and other cellulosic materials. One such application is to chemically attach a dye molecule to a xyloglucan molecule to provide a novel type of dye with high water solubility and excellent substantivity for cotton.

The xyloglucan fragments can either be a collection of intermediate size (S_1 – S_{10}) oligosaccharides, obtained by partial *endo*glucanase digestion or a collection of "S_1" oligosaccharides, made by complete *endo*glucanase digestion of xyloglucan; a dye molecule can be readily linked to the chemically reactive glucose residue at the reducing end of each oligosaccharide. If complete digestion is used, the modified products are then reattached to xyloglucan polysaccharides by the use of an endotransglycosylase [Catalá *et al.* (2001) *Plant Physiol.* 127:1180-1192], as described herein as forming a product that binds avidly to cellulose. The product should contain between 3 and 10 subunits (S_3-S_{10}) to ensure its efficient binding to cellulose (Valent and Albersheim (1974) *supra*; Hayashi *et al.* (1994) *Plant Cell Physiol.* 35:893-899] while keeping its dye content high enough to impart intense color to the cellulose.

Fig. 5 shows the synthetic process of an XGO-dye conjugate. In Method A, tamarind xyloglucan is partially digested with *endo*glucanase (30 min at 25 °C) to give a mixture of xyloglucan oligosaccharides (XGO), which are reductively aminated with an aromatic amine. After dialysis, which removes excess aromatic amine, the product is treated with a diazotized

aromatic amine to give a mixture of XGO-dye conjugates (see Figure 5) most of which are in the mass range between 1 and 10 kDa and can be purified by ultrafiltration.

In Method B, tamarind xyloglucan is exhaustively digested with *endo*glucanase (24 h at 50 °C) to give S_1 XGO. These are reductively aminated with an aromatic amine and then coupled with a diazotized aromatic amine to give an orange S_1-dye conjugate. Reaction of the S_1-dye conjugate with xyloglucan polymer, catalyzed by xyloglucan endotransglycosylase, yields a mixture of XGO-dye. Varying the ratio of xyloglucan to S_1-dye (between 2:1 and 10:1) has a profound influence on the size distribution of the product. The XGO-dye mixture can be fractionated by ultrafiltration.

The xyloglucan polymers are easily obtained from inexpensive and readily available Tamarind seed meal by extraction with water [York *et al.* (1990) *supra*].

Coupling components suitable for covalent attachment to xyloglucan include but are not limited to 5-amino-1-naphthol and 7-amino-4-hydroxy-2-naphthalenesulfonic acid (J-acid).

Azo components suitable for coupling to xyloglucan-arylamine conjugates can be obtained by diazotization of aromatic amines such as aniline, sulfanilic acid, and 3-nitroaniline.

A dye molecule can be attached selectively to the reactive reducing end of a xyloglucan fragment by employing well-established chemical methods in the art, which include, but are not limited to, reductive amination [Lee *et al.* (1991) *Carbohydr. Res.* 214:155-168], oxidation followed by esterification or amide bond formation (Emmerling and Pfannemüller, 1980), or formation of a glycosylamine or aminoalditol followed by amide bond formation, or addition of carbon nucleophiles [Honda *et al.* (1989) *Anal. Biochem.* 180:351-357].

In addition to the above process of performing an azo coupling on a derivatized oligosaccharide, preformed dye molecules can directly be linked to xyloglucan oligosaccharides. In this case, suitable dyes are not limited to azo compounds, but can include anthraquinone, phthalocyanine, and oxazine colorants, as well as stilbene-derived fluorescent brightening agents

[Shore, J. (1990) "Historical Development and Classification of Colorants" In: Colorants and Auxilliarios, Vol. 1 pp. 1-31 (J. Shore, ed.) The Society of Dyers and Colorists, Bradford].

The binding rate of xyloglucan conjugates can be increased by partially removing galactosyl residues with beta-galactosidase. As shown in Table 2, binding efficiency of the beta-galactosidase digest is increased relative to undigested XGO-dye conjugates.

While the foregoing Specification teaches the principles of the present invention, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, or modifications, as come within the scope of the following claims and their equivalents. Moreover, the invention as disclosed herein, may be suitably practiced in the absence of the specific elements, which are disclosed herein.

All references cited in the present application are incorporated by reference herein to the extent that they are not inconsistent with the present disclosure.

EXAMPLES

Example 1:

Partial digestion of xyloglucan with *endo*glucanase and reductive amination with aniline. Tamarind xyloglucan (1.0 g) was dissolved in 100 mL 50 mM acetate buffer (pH 5.0) and treated with 1000 U *endo*-glucanase ("*endo*-cellulase" from Megazyme, Cat. No. E-CELTR). After agitating the mixture for 30 min at 20 °C, 1.0 M acetic acid was added to bring the pH to 3.85, followed by addition of 1.0 mL aniline. The mixture was stirred for 15 min at 70 °C, cooled, treated with 100 mg $NaCNBH_3$, and stirred for 4 h at 70 °C. The solution was dialyzed (MWCO 1000) against 50 mM acetate buffer (pH 5.0) (5×4 L).

Example 2:

Azo coupling of XGO-aniline. The XGO-aniline solution from Example 1 was treated, at 0 °C, with 800 µL diazonium salt suspension (prepared from 173 mg sulfanilic acid with 6 M HCl (500 µL) and 2.5 M $NaNO_2$ (400 µL)), stirred for 18 h at 4 °C, and purified by ultrafiltration (MWCO 3000).

Example 3:

Complete digestion of xyloglucan with *endo*glucanase. Tamarind xyloglucan (1.2 g) was dissolved in 400 mL 20 mM acetate buffer (pH 5.0) and treated with 320 U *endo*-glucanase ("*endo*-cellulase" from Megazyme, Cat. No. E-CELTR). After agitating the mixture for 24 h at 50 °C, 143 mL of 95 % ethanol was added, and the resulting solution was concentrated on a rotary evaporator. After another addition of ethanol, S_1 precipitated and was separated by centrifugation. The pellet was dissolved in water and lyophilized.

Example 4:

Reductive amination of S_1 with aniline and subsequent azo coupling. S_1 (274 mg) was dissolved in 30 mL 50 mM acetate buffer (pH 4.5) and treated with 137 µL aniline. After 30 min at 60 °C, the mixture was cooled to 0 °C and 274 µL 10 % $NaCNBH_3$ in buffer was added. The solution was heated at 70 °C for 4 h and subsequently dialyzed (MWCO 1000) against 50 mM acetate buffer (pH 5.0) (3×4 L). The resulting solution (37 mL) was treated, at 0 °C, with 150 µL diazonium salt suspension (prepared from 173 mg sulfanilic acid with 500 µL of 6 M HCl and 400 µL of 2.5 M $NaNO_2$). The mixture was stirred at 4 °C for 18 h, loaded onto a C_{18} column, and eluted with a MeOH-water gradient (0-50 %). The colored (λ_{max}=448 nm), carbohydrate-containing, anthrone-positive fractions were pooled and lyophilized to give 117 mg S_1-dye conjugate.

Example 5:

Reductive amination of S_1 with 5-amino-1-naphthol and subsequent azo coupling. S_1 (11.2 mg) was dissolved in 800 µL 50 mM acetate buffer (pH 4.5) and treated with a solution of 13.4 mg 5-amino-1-naphthol in 200 µL acetic acid. After 15 min at 70 °C, the mixture was cooled to 0 °C, and 10 µL 10 % $NaCNBH_3$ in buffer was added. The solution was heated at 70 °C for 2 h, cooled, filtered, and purified by C-18 reverse-phase chromatography (elution with 0-50 % gradient water-methanol). The fractions that tested positive for both carbohydrate (anthrone) and arylamine (diazotized sulfanilic acid) were pooled and the resulting solution was treated, at 0 °C, with 10 µL diazonium salt suspension (prepared from 173 mg sulfanilic acid with 6 M HCl (500 µL) and 2.5 M $NaNO_2$ (400 µL)). The mixture was stirred at 4 °C for 18 h, loaded onto a C_{18} column, and eluted with a MeOH-water gradient (0-50 %). The colored (λ_{max}=448 nm), carbohydrate-containing (anthrone) fractions were pooled and lyophilized to give 3.2 mg S_1-dye conjugate.

Example 6:

XET-catalyzed coupling of S_1-dye with xyloglucan. Tamarind xyloglucan (100 mg) was dissolved in 6 mL 50 mM acetate buffer (pH 5.0). A solution of 25 mg S_1-dye (see Example 4) in 1 mL 50 mM acetate buffer (pH 5.0) was added, quickly followed by addition of 4.8 mL XET. After 24 h at 24 °C, the solution was boiled for 5 min, filtered (0.45µm), and passed through two consecutive ultrafiltration membranes (MWCO 10,000 and 5000, respectively). The material that passed through the first membrane but not through the second (retentate) was used to determine the propensity of the S_1-dye conjugate to bind to cotton.

Example 7:

Binding of variously sized XGO-dye molecules to cotton. A 1.0-mL portion of the 5K retentate of XGO-dye was added to 1001 mg cotton (3×3 mm squares) and incubated at various temperatures (25, 45, 65, and 85 °C). Aliquots of 200 µL of the supernatant were removed

periodically and analyzed by size-exclusion chromatography, using the Absorption of light by the dye at 448 nm to determine the amount of each component. The quantity of each different-sized dye peak, which ranged in size (number of S_1-oligosaccharide repeats with a single dye molecule at the reducing end) from S_1-dye to S_7-dye, was determined both before and after exposing the mixture to cotton (results in Table 1).

25 °C	S_1-dye	S_2-dye	S_3-dye	S_4-dye	S_5-dye	S_6-dye	S_7-dye
0.5 h	11%	26%	33%	34%	36%	40%	35%
2 h	9%	32%	46%	47%	48%	51%	52%
4.5 h	6%	34%	54%	57%	58%	60%	60%
24 h	13%	36%	81%	87%	89%	90%	91%

45 °C	S_1-dye	S_2-dye	S_3-dye	S_4-dye	S_5-dye	S_6-dye	S_7-dye
0.5 h	7%	26%	38%	38%	40%	42%	43%
2 h	6%	31%	55%	57%	58%	60%	59%
4.5 h	7%	30%	66%	71%	73%	76%	74%
24 h	3%	25%	86%	95%	97%	97%	97%

65 °C	S_1-dye	S_2-dye	S_3-dye	S_4-dye	S_5-dye	S_6-dye	S_7-dye
0.5 h	6%	26%	44%	45%	47%	47%	51%
2 h	6%	25%	61%	66%	68%	72%	68%
4.5 h	4%	22%	72%	81%	84%	86%	87%
24 h	4%	17%	87%	97%	98%	99%	99%

85 °C	S_1-dye	S_2-dye	S_3-dye	S_4-dye	S_5-dye	S_6-dye	S_7-dye
0.5 h	4%	20%	49%	53%	55%	58%	57%
2 h	3%	18%	64%	76%	79%	79%	82%
4.5 h	3%	15%	73%	89%	92%	94%	94%
24 h	3%	12%	82%	98%	99%	100%	100%

Table 1. Binding of XGO-dye to cotton.

Example 8:

Influence of removing galactosyl residues on binding of XGO-dye to cellulose. A 2.0-mL portion of the 5K retentate from Example 5 was mixed with 40 µL 1.0 M acetate buffer (pH 5.6) and then 16 U β-galactosidase was added. The mixture was incubated at 50 °C. Half of the mixture ("A") was removed after 6.5 h, boiled for 5 min, filtered, and analyzed by size-exclusion chromatography. The remainder ("B") was allowed to react for additional 6.5 h and then

analyzed. Both A and B were incubated with 100 mg cotton at 65 °C. Aliquots of 200 μL of the supernatant were removed periodically and analyzed by size-exclusion chromatography. The areas of the different XGO-dye components, ranging in size from S_1-dye to S_7-dye were compared to those of the mixture before addition of cotton (see Table 2). Since there was no clear separation between the individual components, the area of peaks with retention times between S_3 and S_7 were summed (see column in Table 2 labled XGO-dye).

	A	B	XGO-dye
0.5 h	64%	72%	45%
2 h	89%	92%	65%
4.5 h	98%	98%	78%
24 h	99%	100%	93%

Table 2. Binding of galactosidase-treated XGO-dye to cotton.

The further details of the invention is provided in Appendix I attached herewith.

REPRESENTATIVE CLAIMS:

1. A method of making a xyloglucan conjugate comprising the steps of:

   (a) preparing xyloglucan fragments from xyloglucan polymers; and

   (b) attaching a functional group to the reducing end of the xyloglucan fragments whereby a xyloglucan conjugate useful for binding to cellulosic material is produced.

2. The method of claim 1 wherein said xyloglucan fragments are prepared by enzymatic digestion.

3. The method of claim 2 wherein said enzymatic digestion is carried out by employing *endo*glucanase.

4. The method of claim 1 wherein said xyloglucan fragments are a mixture of oligosaccharides ranging in size from two to one hundred glycosyl residues.

5. The method of claim 4 wherein said xyloglucan fragments are a mixture of oligosaccharides ranging in size from two to twenty glycosyl residues.

6. The method of claim 1 wherein said xyloglucan fragments range in size from 1 to 10 single tetraglucoside backbones linked together.

7. The method of claim 6 wherein said xyloglucan fragments consist of a single tetraglucoside backbone.

8. The method of claim 1 wherein said functional group is a dye molecule.

9. The method of claim 8 wherein said dye molecule is an azo dye.

10. The method of claim 1 wherein said functional group is a fabric softener.

11. The method of claim 1 wherein said functional group is a lubricant.

12. The method of claim 1 wherein said functional group is an antimicrobial compound.

13. The method of claim 1 wherein said functional group is a water repellant.

14. The method of claim 1 wherein said functional group is an oil repellant.

15. The method of claim 1 wherein said functional group is a firming agent.

16. The method of claim 1 wherein said functional group is an aromatic amine.

17. The method of claim 1 wherein said functional group is attached in a 2-step process comprising (i) attaching an aromatic amine and (ii) performing an azo coupling on the resulting carbohydrate conjugate.

18. A xyloglucan conjugate capable of binding to cellulosic material.

19. The xyloglucan conjugate of claim 18 comprising a dye molecule.

20. The xyloglucan conjugate of claim 19 wherein said dye is an azo dye.

21. The xyloglucan conjugate of claim 18 comprising a fabric softener.

22. The xyloglucan conjugate of claim 18 comprising a fluorescent brightening agent.

23. The xyloglucan conjugate of claim 18 comprising a lubricant.

24. The xyloglucan conjugate of claim 18 comprising an antimicrobial compound.

25. The xyloglucan conjugate of claim 18 comprising a water repellant.

26. The xyloglucan conjugate of claim 18 comprising an oil repellant.

27. The xyloglucan conjugate of claim 18 comprising a firming agent.

28. The xyloglucan conjugate of claim 18 wherein the cellulosic material is cotton.

29. A method of attaching a functional group to cellulosic material comprising the steps of:

(a) preparing xyloglucan fragments from xyloglucan polymers by hydrolysis;

(b) attaching the functional group to the reducing end of the xyloglucan fragments to produce a xyloglucan conjugate; and

(c) treating a cellulosic material with the xyloglucan conjugate whereby the cellulosic material containing the xyloglucan conjugate is produced.

30. The method of claim 29 wherein said functional group is a dye molecule.

31. The method of claim 29 wherein said functional group is a lubricant.

32. The method of claim 29 wherein said functional group is a fluorescent brightening agent.

33. The method of claim 29 wherein said functional group is a fabric softener.

34. The method of claim 29 wherein said functional group is an antimicrobial compound.

35. The method of claim 29 wherein said functional group is a water repellant.

36. The method of claim 29 wherein said functional group is an oil repellant.

37. The method of claim 29 wherein said functional group is a firming agent.

38. The molecule of claim 30 wherein said dye molecule is an azo dye.

39. The method of claim 29 wherein said cellulosic material is cotton.

40. The method of claim 29 wherein said hydrolysis step is carried out by using an enzyme selected from the group consisting of beta-galactosidase, *endo*glucanase, and xyloglucan *endo*transglycosidase (XET).

41. The method of claim 40 wherein said enzyme is XET.

42. The method of claim 29 wherein said xyloglucan fragments consist of a single tetraglucoside backbone.

43. The method of claim 29 wherein said xyloglucan conjugates are treated with beta-galactosidase

44. The method of claim 40 wherein said aromatic amine is attached by reductive amination.

45. The method of claim 40 wherein said aromatic amine is attached by electrolytic oxidation, followed by amide bond formation.

46. The method of claim 40 wherein said aromatic amine is attached by carbon-carbon bond formation between xyloglucan fragments and a heterocyclic compound.

47. The method of claim 46 wherein said heterocyclic compound is a pyrazolinone derivative.

48. The method of claim 29 wherein said xyloglucan fragments are purified by ultrafiltration.

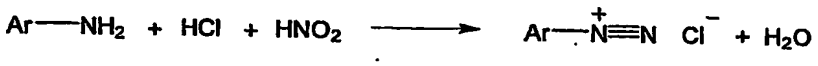
49. The method of claim 29 wherein said xyloglucan conjugates are purified by ultrafiltration.

ABSTRACT

The present invention provides xyloglucan conjugates useful as molecular anchors for attaching various functional chemical groups to cellulose or cellulosic materials. The functional groups in the xyloglucan conjugates can serve as dyes, fabric softeners, antimicrobial agents, and flame retardants and the like. Also provided are methods of preparing and using the xyloglucan conjugates of the invention.

Synthesis of azo dyes.

Diazotization



Azo coupling

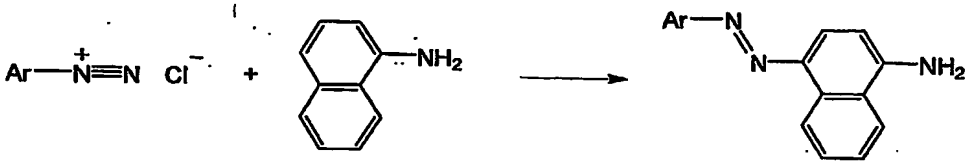
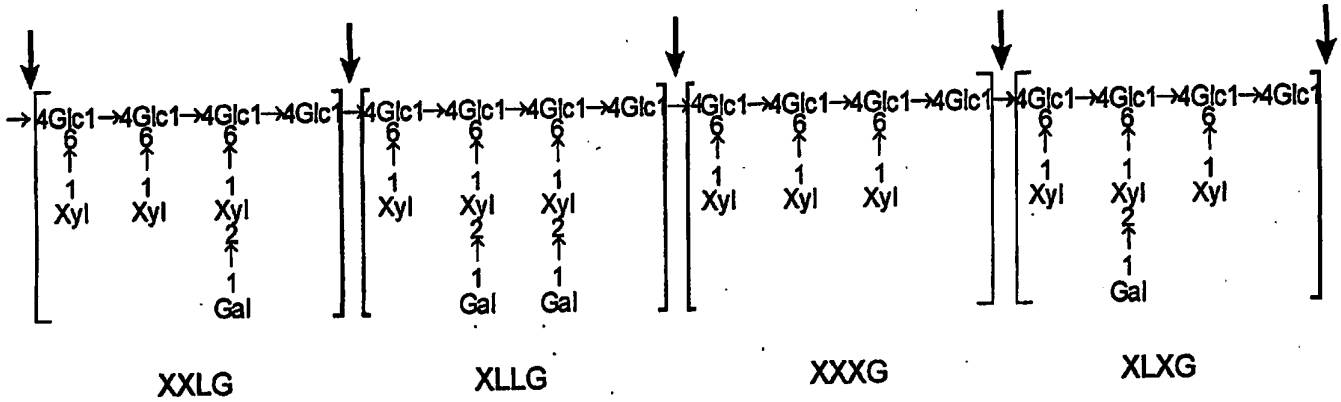


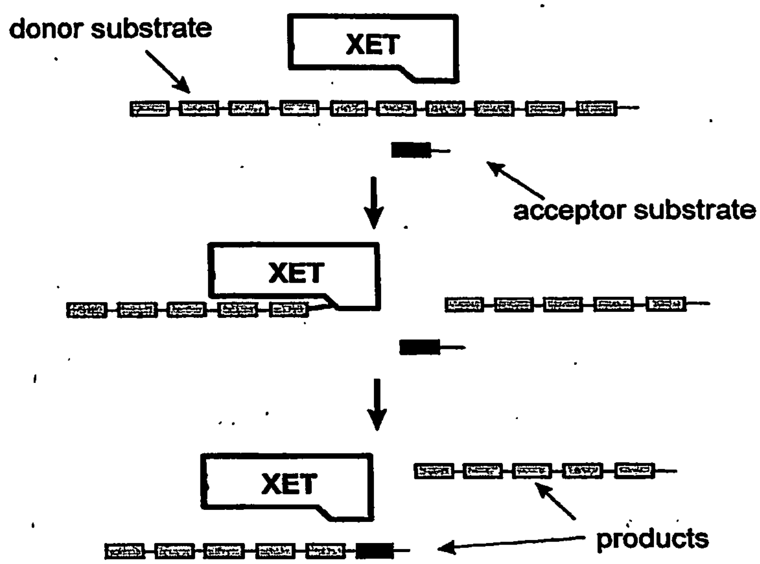
FIG. 1



Structure of Tamarind Xyloglucan.

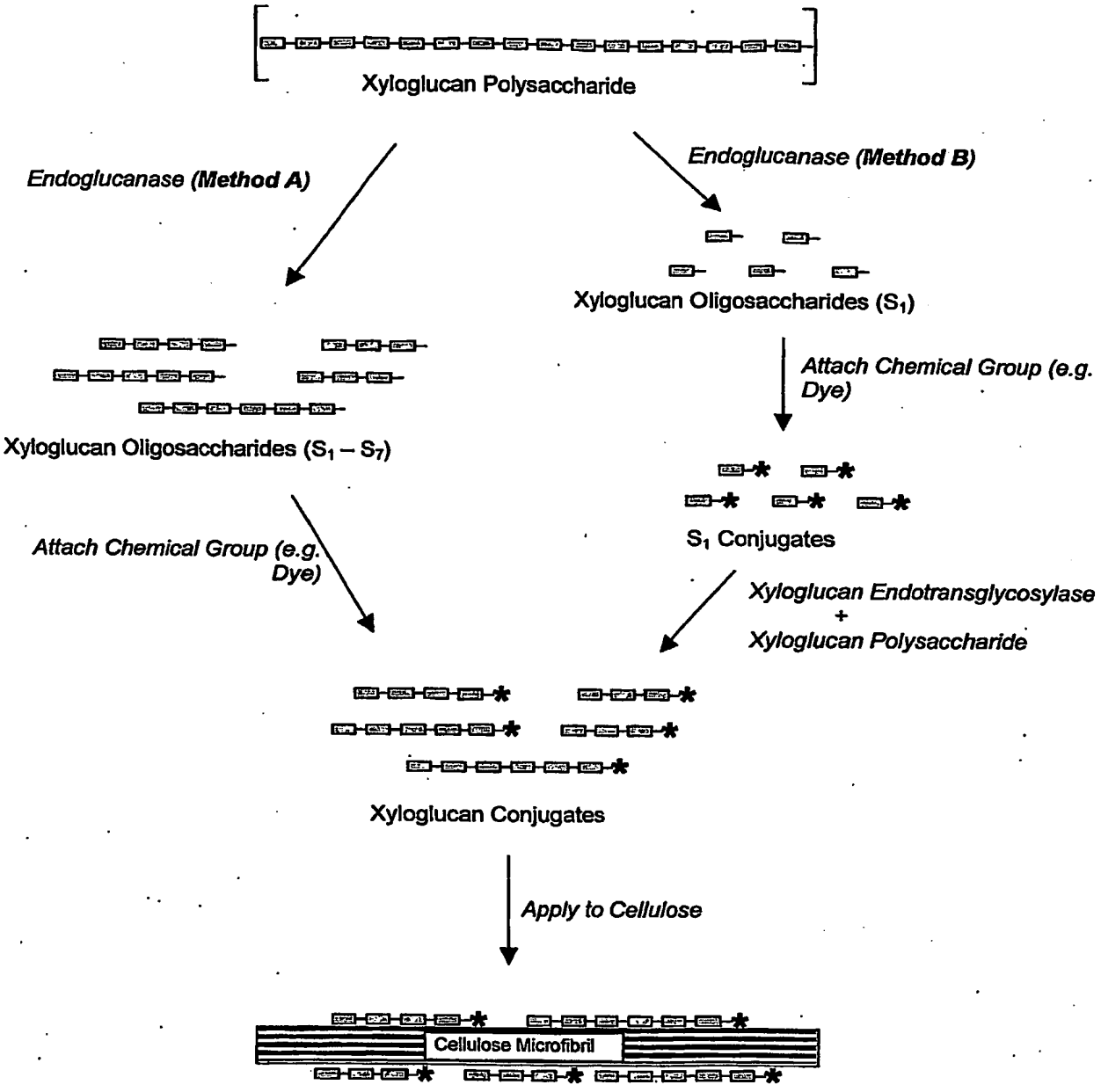
Arrows indicate glycosidic bonds that are susceptible to attack by endoglucanase or xyloglucan endotransglycosylase

FIG. 2



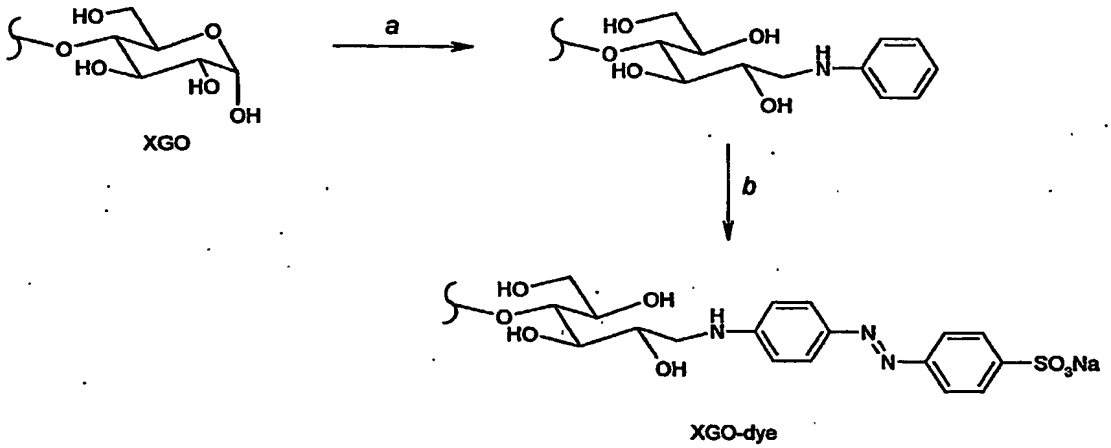
Action of Xyloglucan Endotransglycosylase.
Two different xyloglucan substrates are distinguished by their shading. Each oligosaccharide subunit is indicated by a rectangle

FIG. 3



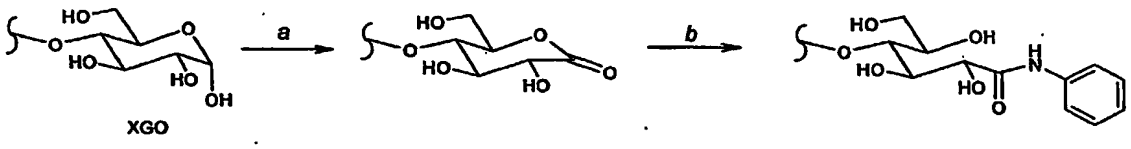
Preparation and Use of Xyloglucan Conjugates
Xyloglucan Subunit oligosaccharides are indicated by rectangles.
Reducing ends are indicated by a dash on the right side of each molecule.
Dye or other attached molecules are indicated by asterisks.

FIG. 4



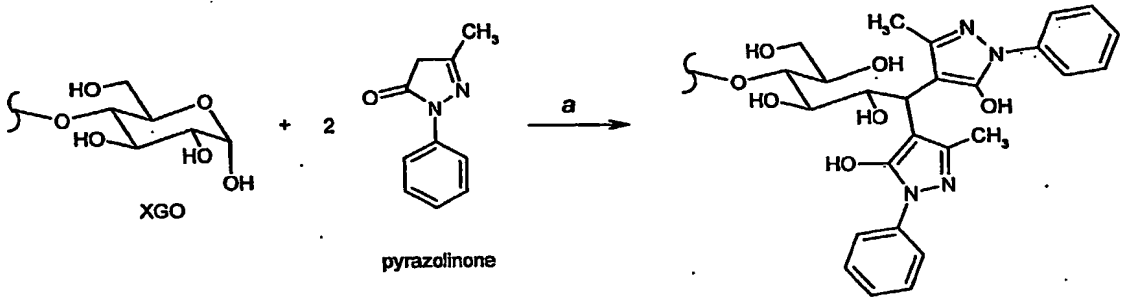
Synthesis of XGO-dye conjugates.
Reaction conditions: a. aniline, $NaCNBH_3$, 70 °C, 3h; b. diazotized sulfanilic acid, 0-5 °C, 18 h.

FIG. 5



Electrolytic oxidation of XGO and amide bond formation.
Reaction conditions: a. $CaBr_2$, $CaCO_3$, graphite electrodes, 4.5 V, 25 °C, 3 h; b. aniline.

FIG. 6



Condensation of XGO with pyrazolinones.
Reaction conditions: a. NaOH, EtOH, 60 °C, 2h.

FIG. 7